PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/18, C07K 1/00

A1

(11) International Publication Number: WO 97/31647

(43) International Publication Date: 4 September 1997 (04.09.97)

(21) International Application Number: PCT/US97/03520

(22) International Filing Date: 3 March 1997 (03.03.97)

(30) Priority Data:
08/609,533 1 March 1996 (01.03.96) US
08/612,719 8 March 1996 (08.03.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US Not furnished (CIP)
Filed on Not furnished

(71) Applicants *(for all designated States except US)*: IMCLONE SYSTEMS INCORPORATED [US/US]; 180 Varick Street, New York, NY 10014 (US). TRUSTEES OF PRINCETON UNIVERSITY [US/US]; Princeton University, Princeton, NJ 08544 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WITTE, Larry [US/US]; R.D. 2 Box 740 Williams Cross Road, Stormville, NY 12582 (US). PYTOWSKI, Bronislaw [US/US]; Apartment F2, 583 West 215th Street, New York, NY 10034 (US). MOORE, Kateri, A. [US/US]; 248 Hawthorne Avenue, Princeton, NJ 08540 (US). LEMISCHKA, Ihor, R. [US/US]; #4 Firestone Court, Princeton, NJ 08540 (US).

(74) Agent: FEIT, Irving, N.; Hoffmann & Baron, 350 Jericho Turnpike, Jericho, NY 11753 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

(54) Title: USE OF DELTA-LIKE PROTEIN TO INHIBIT THE DIFFERENTIATION OF STEM CELLS

(57) Abstract

A method for inhibiting the differentiation of stem cells is disclosed. The differentiation of stem cells is inhibited by contacting the stem cells with a sufficient amount of a delta-like protein (dlk).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

USE OF DELTA-LIKE PROTEIN TO INHIBIT THE DIFFERENTIATION OF STEM CELLS

FIELD OF THE INVENTION

The present invention is directed to the use of Delta-like protein (dlk) to inhibit the rate of differentiation of stem cells.

BACKGROUND OF THE INVENTION

The differentiated cells of some biological systems mature in stages from a common progenitor cell, usually called a stem cell. Such cells include, for example, hematopoietic, neural, epithelial, endothelial, and mesodermal cells.

Stem cells are able to differentiate into mature cells within one of these systems. Differentiation may occur through uncommitted or committed progenitor cell intermediates.

The differentiation of a stem cell may result in one mature cell. Alternatively, a stem cell may differentiate into multiple mature lineages within a biological system, in which case the stem cell is said to be pluripotent. In

some cases. the differentiation of a stem cell may result in all the mature lineages of an entire biological system, in which case the stem cell is said to be totipotent. Stem cells are also able to self-renew. The self-renewal must be delicately balanced by differentiation in order to maintain a healthy level of stem cells.

For example, hematopoietic stem cells constitute approximately 0.01% of the cells in adult bone marrow. These stem cells. which can be recognized by the presence of the CD34 antigen. are found in micro-environments associated with stromal cells.

Hematopoietic stem cells are induced by various cytokines. such as c-kit and flk-2/flt-3 ligand. to differentiate into increasingly lineage-committed progenitors. These progenitors differentiate further into the various mature white blood cells, red blood cells. and platelets of the hematopoietic system.

Self-renewal of the stem cells requires their maintenance in an undifferentiated state. There are various applications for methods that increase the time during which stem cell populations remain undifferentiated in vivo and ex vivo. Such applications include. for example. gene therapy. and concentrating stem cell populations.

Another application for maintaining stem cells in an undifferentiated state is cell and tissue transplantation between animals. An example of this application, in the case of hematopoietic cells, is bone marrow transplantation.

Attempts to maintain and expand stem cells, especially hematopoietic stem cells, using various cytokines and cytokine mixtures have been unsuccessful. An object of the present invention is to provide methods for the

maintenance of stem cells in an undifferentiated state. A more specific object is to provide methods for the maintenance of hematopoietic stem cells in an undifferentiated state. Another object is to provide a method for enriching a population of stem cells in a mixture of stem cells and non-stem cells.

SUMMARY OF THE INVENTION

These, and other, objects as will be apparent to those having ordinary skill in the art have been met by providing a method for inhibiting the differentiation of stem cells, such as hematopoietic stem cells: and a method for enriching a population of stem cells in a mixture of stem cells and non-stem cells. The methods comprise contacting the stem cells, or the mixture of stem cells and non-stem cells, with a sufficient amount of human delta-like protein (dlk).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for inhibiting the differentiation of stem cells. For the purpose of this application, inhibiting the differentiation of stem cells means preventing, or inhibiting the rate of, the differentiation of stem cells.

A stem cell refers to any cell that is capable of self renewal, and of differentiating into committed progenitors of one or more lineages within a group of cells of a biological system. Such cells include, for example, hematopoietic, neural, epithelial, endothelial, and mesodermal cells. Preferably, the stem cells are capable of re-populating at least one lineage, preferably

multiple lineages, and more preferably all lineages of a biological system of cells in an ablated mammal. The method involves contacting the stem cells in vivo or ex vivo with a sufficient amount of delta-like protein (dlk).

The dlk protein and gene is described by Laborda et al. in the Journal of Biological Chemistry 268, 3817-3820 (1993); Lee et al. in Biochimica et Biophysica Acta 1261, 223-232 (1995); and Laborda, International PCT Application WO 94/13701. The dlk gene and protein can be isolated and purified as described in the Laborda et al. and Lee et al. articles and in the Laborda patent application.

The dlk gene and protein may originate from any mammalian source. The human gene and protein are preferred. The mouse and human dlk amino acid sequences are given in Figures 1A and 1B, respectively, of the Laborda patent application; the sequence of mouse and human dlk nucleotide sequence is given in Figures 3 and 2, respectively, of the Laborda patent application; and the amino acid and nucleotide sequences of human dlk are given in Figure 6 of the Lee et al. article. These sequences are incorporated herein by reference.

The dlk gene and protein useful in the present invention may contain mutations and polymorphisms in the sequences as described above. Preferably, the dlk gene and protein are at least about 75% identical, preferably at least about 85%, more preferably at least about 95%, and most preferably at least about 99% identical to the native nucleotide or amino acid sequence of a mammal, such as of the mouse and human sequences described above.

As shown in Figure 2 of the Laborda et al. article, dlk is a transmembrane protein that contains six EGF-like repeats in an N-terminal extracellular domain and a short C-terminal intracellular domain separated by

the transmembrane domain. Figure 2 of the Laborda et al. article is incorporated herein by reference.

The dlk protein useful in the present invention may be a full length protein. Alternatively, the dlk may be in a soluble form (sdlk). The soluble protein may comprise the entire extracellular domain, or at least enough of the extracellular domain for the molecule to retain its biological activity. The extracellular domain should comprise at least three EGF repeats (for example, EGF repeats 1-3, 2-5, or 3-6); preferably at least four EGF repeats (for example, EGF repeats 1-4, 2-5, or 3-6); more preferably five EGF repeats (for example, EGF repeats 1-5, or 2-6); and most preferably all six EGF repeats. The EGF repeats are shown in Figure 2 of the Laborda et al. article, which is incorporated herein by reference.

The soluble dlk protein preferably lacks both the transmembrane domain and the intracellular domain. Alternatively, the soluble dlk protein may contain all or part of the transmembrane domain and either no part of the intracellular domain, or as much of the intracellular domain that still permits the dlk protein to retain its solubility in water at room temperature.

Soluble forms of dlk can be produced by methods known in the art. For example, the soluble dlk protein may be produced by cloning an appropriate DNA sequence in a suitable expression vector. The DNA is transfected into a suitable host cell, such as, for example, COS, CHO, NIH 3T3, and expressed.

Optionally, the soluble dlk protein is expressed as a fusion protein. Examples of fusion protein partners to be expressed in tandem with the dlk protein include, but are not limited to, IgG protein, metallotheonine, histidine repeats, alkaline phosphatase and FLAG protein.

The dlk protein is then expressed, and preferably secreted directly into the cell media. The protein is harvested, preferably in serum-free media, from the stable expressing cell lines.

The soluble protein can be purified by methods known in the art. A suitable purification method is affinity chromotography.

The purified protein is used for any of the utilities described in the specification. The protein can also be used to immunize animals for the production of anti-dlk monoclonal or polyclonal antibodies. Such antibodies can be used to identify and isolate dlk-containing cells, such as stromal cells, that express the dlk protein.

The sdlk may be used in solution. Alternatively, the sdlk may be aggregated by physical means, such as, for example, heat, pH, etc., or chemical means, such as chemical cross linking, as is well known in the art. Such "multivalent" forms of sdlk are better able to cross link the appropriate target molecules on the surface of stem cells, and to provide the appropriate biological signals. The sdlk molecules may also be coated on small, inert beads, such as latex beads for example, or on the surface of a container, such as a culture dish.

If the dlk protein used in the method of the invention contains the transmembrane region, the dlk protein may be attached to the surface of a cell, or may be independent of cells. Some cells, such as some stromal cells, contain surface dlk naturally. An example of such a cell is the stromal cell line known as AFT024.

Alternatively, the cells may not contain surface dlk naturally, but may be transfected with the dlk gene so as to express one or more surface dlk proteins. Some examples of cells suitable to be transfected with the dlk gene include COS, CHO, NIH 3T3, and BFC012.

The dlk protein and its variants, as described above, prevents, or at least inhibits the rate of, the differentiation of hematopoietic stem cells. For this purpose, the dlk protein may be attached to the surface of a stromal cell. Alternatively, the dlk protein may be attached to the surface of a non-stromal cell or may be independent of a cell, in which case it is preferably used in conjunction with stromal cells or other factors, such as those that exist on stromal cells, that, in conjunction with dlk, prevent, or at least inhibit the rate of, the differentiation of hematopoietic stem cells.

The dlk protein and its variants, as described above, may also be used along with a cocktail of growth factors. The cocktail, including the hematopoietic cocktail described below, may be replaced by, or used in conjunction with, 5-fluorouracil.

The growth factors are selected for inclusion in the cocktail so as to be specific for the type of stem cells that are being maintained in an undifferentiated state. The cocktail has the effect of enhancing the inhibition of differentiation, and of inducing the proliferation of the stem cells, leading to self-renewal.

For example, a cocktail of growth factors for hematopoietic stem cells comprises a mixture of one or more of IL-1, G-CSF, GM-CSF, c-kit ligand, IL-3, IL-6, and flk-2/flt-3 ligand. Such cocktails have been described by Moore, et al. in "Ex Vivo Expansion of $CD34^+$ Hematopoietic Progenitors, in Gross, eds.;

Advances in Bone Marrow Purging and Processing. Proceeding of the Fourth International Symposium on Bone Marrow Purging and Processing. Orlando, Florida. Wiley-Liss (1994).

The components of the cocktail, the 5-fluorouracil, and the dlk may be used at any concentration at which they are effective. The components of the cocktail and the 5-fluorouracil may, for example, be suitably used at concentrations of 0.1-100 ng/ml, preferably 1-50 ng/ml, and more preferably 5-20 ng/ml. The dlk may, for example, be suitably used at concentrations of 1 ng-100 ug/ml, preferably 0.1-100 ug/ml, more preferably 1-50 ug/ml, and most preferably 5-20 ug/ml.

The dlk protein and its variants as described above may be used on its own, or in conjunction with a cocktail of growth factors, such as a cocktail of hematopoietic growth factors, and/or 5-fluorouracil to prevent, or at least to inhibit the rate of, differentiation ex vivo. The stem cells may be found in a mixture of stem cells and non-stem cells. For example, a mixture of hematopoietic stem cells and more mature, non-stem cells occurs in peripheral blood, bone marrow or umbilical cord blood.

During the increased time the stem cells in the mixture are maintained by the dlk in an undifferentiated state, other, more mature, non-stem cells continue to differentiate, and ultimately die. In this way, the hematopoietic stem cells become enriched and concentrated in the mixture of stem cells and non-stem cells.

For example, a mixture of hematopoietic stem cells, preferably from the bone marrow, may be removed from a patient undergoing chemotherapy or

radiation treatment. Patients who will benefit especially are those being treated for cancers. such as those of the white blood cells. i.e. leukemia.

For example, the patient's bone marrow is removed from the patient. The bone marrow is purged of any malignant cells. and the patient is subjected to chemotherapy or radiation treatment. The chemotherapy or radiation treatment depletes the patient's hematopoietic cells.

The hematopoietic stem cells are maintained ex vivo in an undifferentiated state with dlk. as described above. The stem cell population may be expanded at this stage.

Alternatively, the stems cells may be isolated using either negative or positive selection. An example of positive selection is the separation of a stem cell fraction from a larger cell fraction by binding the stems cells to a monoclonal or polyclonal antibody specifc to a stem cell antigen, such as CD34. Such antibodies may be bound to microbeads or to a column matrix in order to facilitate the isolation of the stem cells.

An example of negative selection includes passing a cell fraction through a column in which a variety of antibodies directed against mature cell antigens are used to bind the more mature cells. allowing the stem cells lacking these antigens to be separated into a specific fraction. The antibodies may be monoclonal or polyclonal.

Following treatment. the stem cells are reinfused into the patient for self-renewal of the patient's hematopoietic system. The stem cells are preferably derived from the patient undergoing chemotherapy or radiation treatment. If necessary, the stem cells may be derived from a patient other than the patient

undergoing chemotherapy or radiation treatment. The patient is a mammal, preferably a human.

Alternatively, the stem cells may be removed from a patient and subjected to gene therapy while the cells are maintained in an undifferentiated state by the dlk protein, or a variant, as described above. The presence of the dlk increases the length of time a stem cell is available for gene therapy.

In vivo, the dlk, preferably sdlk, may be used to expand the available pool of hematopoietic stem cells. Such treatment is of benefit before chemotherapy or radiotherapy for various malignancies. Increased stem cell populations would allow a more rapid re-population of all blood cells, including leukocytes and platelets, that are typically depleted during such therapies.

dlk may be administered to patients by methods known in the art. Preferably, sdlk is administered by intravenous injection, or is injected directly into the bone marrow cavity.

EXAMPLES

Example 1. Soluble dlk

1. A full-length cDNA clone for human dlk (hdlk) was obtained as described above. A soluble dlk (sdlk) expression construct was prepared by PCR by truncating the cDNA before the first codon of the predicted transmembrane domain. The positive (5') primer (Primer 1) was located starting eight nucleotides 5' of the ATG codon, and encoded a Hind III restriction site. The negative (3') primer (primer 2) introduced a stop codon and

a Not I site immediately following the codon for the ultimate alanine in the extracellular domain of human dlk. The sequences of these primers are given below. The resulting PCR fragment was cloned into the eukaruotic expression vector pcDNA3 (Invitrogen Inc.). Protein expression was achieved by introducing the expression plasmid into NIH 3T3 and CHO cells and establishing permanent lines of expressing cells. In parallel, constructs directing the expression of sdlk were joined to sequences expressing the coding regions for alkaline phosphatase and for the Fc region of human IgG1. The resulting fusion proteins (sdlk-AP and sDlk-Ig) were expressed as indicated above for sdlk. These fusion proteins can be easily purified and used in several types of binding and functional assays.

Primer 1: 5'-GAG.GGT.ACC.AAG.CTT.CCA.GAG.ATG.ACC.GCG.ACC.GA (SEQ ID NO 1)
Primer 2: 5'GCA.TCT.AGA.GCG.GCC.GCT.CAG.GCC.TGT.CCC.TCG GTG.AGG.AG (SEQ ID NO 2)

Example 2 Cell lines and culture

The AFT024 and BFC012 stromal cell lines were characterized for their ability to support highly enriched fetal liver and adult bone marrow stem cells using both *in vitro* and *in vivo* assays. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 mM b-mercaptoethanol (BME), and maintained at 31^0-33^0 C, 5% CO_2. For long-term cocultures with hematopoietic stem cells, confluent monolayers were irradiated (20 Gy), placed in modified Dexter media (DMEM, 10% FBS, 10% horse serum, 50 mM BME, 0.1 mM hydrocortisone) and maintained at 37^0 C, 5% CO_2 with weekly media changes. NIH 3T3 cells were obtained from ATCC.

Example 3: dlk expression analysis

Total RNAs from stromal cell lines were poly A+ selected, Northern blotted, and hybridized to ^{32}P-labeled probes according to standard protocols. A 600 bp dlk cDNA clone from the AFT024 subtracted library was used as a probe. cDNA templates for RT-PCR were prepared using SuperScript reverse transcriptase according to manufacturer's protocols (GibcoBRL). Oligonucleotide primers for the dlk PCR reactions were: sense 5'-GACCCAGGCTGCCCC-3' (SEQ ID NO 3) and antisense 5'-GGTACTCTTGTTGAG-3' (SEQ ID NO 4). For analysis of dlk expression at the protein level, antisera specific for dlk was generated by immunizing rabbits with a Flag-dlk fusion protein (described below). Resultant antibodies were purified by affinity chromatography on Sepharose Cl-4B to which dlk-Ig (described below) was coupled according to the manufacturer's instructions (Pharmacia Biotech Inc.). After elution and neutralization, affinity purified dlk antibodies were dialyzed against phosphate buffered saline (PBS) and protein concentration was determined by the BCA method (Pierce). Cell surface expression of dlk in native and transfected (see below) stromal cell lines was accomplished by flow cytometry with the affinity purified dlk antibody. Stromal cell lines in active growth phase were washed once with PBS/0.5 mM EDTA, and harvested by trituration in PBS/EDTA. Twice washed (PBS/3%FBS) cells were incubated with dlk antibody and a similarily prepared irrelevant control antibody. Specific labeling was developed by donkey anti-rabbit-FITC (Jackson Immunoresearch). Stained cells were analyzed on a Becton Dickinson FACScan using Cell Quest software.

Example 4 dlk fusion protein preparation

The expression plasmid pCD4-Ig contains cDNA for the extracellular domain of human CD4 fused to genomic sequences of the human immunoglobin heavy chain. This cDNA was cloned into *Eco*RI and *Not* I sites of pcDNA3 (Invitrogen) to give the plasmid KB52.3.2. cDNA encoding the extracellular domain of dlk was obtained by RT-PCR with primers BP 151 and BP 152 using total RNA from NIH 3T3 cells as template. The primer BP 152 includes the last codon in the dlk extracellular domain and contains an *Eco*RI site in frame with that of KB52.3.2. The resulting PCR fragment was cloned into KB52.3.2 via *Hind* III and *Eco*RI sites to obtain the soluble dlk-Ig expression plasmid. pdlk-Ig or pCD4-Ig were transfected into NIH 3T3 cells together with pSVNeo and stable clones were isolated after selection in 400 mg/ml G418 (active wt., GibcoBRL) in DMEM, 10% FBS. Primers: sense BP 151: 5'GAGGGTACCAAGCTTCGTGGTCCGCAACCAGAAG-3' (SEQ ID NO 5); anti-sense BP 152: 5'-CTCAGATCTGAATTCGGCCTGTCCCTCGG TGAGGAG-3' (SEQ ID NO 6).

The CD4-Ig and dlk-Ig proteins were harvested in serum-free media from stable expressing cell lines. The soluble fusion proteins were purified by affinity chromatography on HiTrap Protein G-sepharose (Pharmacia Biotech Inc.) equilibrated with 0.1 M Tris-HCl, pH 7.6, 0.5 M NaCl and eluted with 0.2 M glycine-HCl, pH 2.8, 0.5 M NaCl. Eluted protein was dialyzed against PBS. Protein concentration was determined by the BCA method (Pierce).

Flag-dlk fusion protein was used to immunize rabbits for the production of dlk antiserum. The protein expression plasmid pcDNA3-Flag is a modification of the plasmid pcDNA3 (Invitrogen) and contains 22 base pairs of 5' untranslated sequence, a preprotrypsinogen signal sequence, and the coding

region for the Flag peptide (DYKDDDDKI) as well as a *Bgl* II restriction site. A cDNA fragment encoding the extracellular domain of dlk was obtained by RT-PCR using RNA from NIH3T3 cells. The 5' primer (BP155) was designed to introduce an in frame *Bgl* II site at the 5' end of the predicted mature dlk protein coding sequence. The 3' primer (BP154) contained *Xba* I and *Not* I sites downstream of a stop codon which is immediately adjacent to the last amino acid of the predicted dlk extracellular domain. Primers: sense BP 155: 5'-GACAAGATCTCAGCTGAATAGCGACCCACCCTGTG-3' (SEQ ID NO 7); antisense BP 154: 5'-GCATCTAGAGCGGCCGCTCAGGCCTGTCCCTCGGTGAGGAG-3' (SEQ ID NO 8). The PCR fragment was digested with *Bgl* II and *Not* I and ligated into pcDNA3-Flag to yield pFlag-dlk. pFlag-dlk was transfected into COS cells using DEAE-dextran. Affinity purification of the Flag-dlk protein from COS conditioned media was performed according to manufacturer's directions using the Flag monoclonal antibody, M1, immobilized on agarose (International Biotechnologies).

Example 5 Plasmid constructs and stable transfection

Full-length murine dlk cDNA was obtained by RT-PCR with primers BP 151 (SEQ ID NO 5, see above) and antisense BP 200: 5' GCATCTAGAGCGGCCGCGAACGCTGCTTAGATCTCCT-3' (SEQ ID NO 9), using total RNA from NIH3T3 cells as template. Sequencing confirmed that the resulting product was identical to the published dlk sequence. The product was subcloned into the vector pCRII (Invitrogen) and then cloned into a retroviral expression vector via the primer-encoded *Hind*III and *Not*I sites. Supercoiled plasmid was transfected into BFC012 stromal cells by the calcium phosphate method, according to the manufacturer's protocol (GibcoBRL) together with the pZeo (Invitrogen) selectable marker and selected in 50 mg/ml

Zeocin (Invitrogen). BFC012 cells were also transfected with pZeo alone and selected as above. Clones from both selected populations were isolated and all remaining colonies (100-200/dish) were pooled and expanded as populations.

Example 6 Hematopoietic stem cells and in vitro hematopoietic assays.

Hematopoietic stem cell populations were derived from wild type, Ly5.2-C57Bl/6J (Jackson Laboratories), day 14 fetal liver, enriched for the AA4.1^{+}, Sca-1^{+}, c-kit^{+}, and lin$^{lo/-}$ phenotype, by immunopanning and fluorescence-activated cell sorting. Adult bone marrow (BM) was used directly after density centrifugation and immunomagnetic bead depletion or was further enriched for Sca-1^{+}, c-kit^{+}, lin$^{lo/-}$ cells by flow cytometry as described. Cell sorting and data analysis was accomplished with a Becton Dickinson FACS Vantage using Cell Quest Software. Stromal cell/stem cell cocultures were initiated in 12-well trays with 300-1000 enriched stem cells per well. Cobblestone areas were quantitated by inverted-phase microscopy as described. Clonogenic progenitor assays were performed with either freshly purified stem cells or cells harvested from the stromal cocultures. These were cultured in cytokine-containing semisolid media according to the manufacturer's recommendations (Stem Cell Technologies). Progenitor colonies were scored after 8-12 days according to established criteria. Soluble dlk and control fusion proteins were added to semisolid progenitor assays at concentrations of 0.1, 0.5 and 1.0 mg/ml and also to BFC012 stromal cocultures at concentrations of 0.1 mg/ml. Fusion protein was replenished weekly in the stromal cocultures.

Example 7 Competitive repopulating transplantation assay

Cultured cells were harvested, combined with fresh unfractionated BM obtained from congenic C57Bl/6 Ly5.1 mice (National Cancer Institute) and transplanted into lethally irradiated (10 Gy, split dose 3 hours apart from a ^{137}Cs source, 1 Gy/min) Ly5.1 recipient mice. Each mouse received 2 X 10^5 competitor BM cells and a fraction of the cocultured stem cells. Mice were bled by capillary puncture of the orbital venous plexus and 100 ml were collected into heparin-containing (10 U/ml) DMEM: red blood cells were removed by NH_4Cl lysis. The nucleated cells were stained for the Ly5.2 (CD45.2) allelic marker using either FITC-labeled directly conjugated Ly5.2 monoclonal antibody or a biotinylated form developed with streptavidin conjugated to Texas Red. Cells were also stained with directly conjugated antibodies to lineage markers. All antibodies and chromogens were obtained from Pharmingen. Flow cytometric analysis was done on a Becton Dickinson FACS Vantage using Cell Quest Software.

SUPPLEMENTAL ENABLEMENT

The invention as claimed is enabled in accordance with the specification and readily available references and starting materials.

Nevertheless, on March 3, 1997, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) dlk-transfected cell line BFC012. This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for thirty years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC, which assures unrestricted availability

upon issuance of the pertinent U.S. patent Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

WE CLAIM:

1. A method for inhibiting the differentiation of stem cells, comprising contacting the stem cells with a sufficient amount of dlk protein.

2. A method according to Claim 1 wherein the stem cells are totipotent.

3. A method according to Claim 1 wherein the stem cells are pluripotent.

4. A method according to Claim 1 wherein the stem cells are neural, epithelial, endothelial, or mesodermal stem cells.

5. A method according to Claim 1 wherein the stem cells are hematopoietic stem cells.

6. A method according to Claim 1 wherein the dlk protein is human dlk protein.

7. A method according to Claim 1 that takes place ex vivo.

8. A method according to Claim 7 wherein the stem cells are hematopoietic stem cells.

9. A method according to Claim 8 wherein the hematopoietic stem cells are in the peripheral blood, bone marrow, or cord blood of a human.

10. A method according to Claim 1 wherein the dlk is used in conjunction with a cocktail of growth factors.

11. A method according to Claim 5 wherein the dlk is used in conjunction with a cocktail of growth factors for hematopoietic stem cells.

12. A method according to Claim 1 wherein the dlk is used in conjunction with 5-fluorouracil.

13. A method according to Claim 5 wherein the dlk is used in conjunction with 5-fluorouracil.

14. A method according to Claim 1 wherein the dlk protein is independent of a cell.

15. A method according to Claim 14 wherein the dlk protein is soluble dlk protein.

16. A method according to Claim 1 wherein the dlk protein is attached to the surface of a cell.

17. A method according to Claim 16 wherein the cell is a stromal cell.

18. A method according to claim 17 wherein the stromal cell is BFC012 transfected with the dlk gene.

19. A method according to claim 17 wherein the stromal cell is AFT024.

20. A method for enriching a population of stem cells in a mixture of stem cells and non-stem cells comprising contacting the mixture with a sufficient amount of human dlk protein.

21. A method according to claim 20 wherein the stem cell are hematopoietic stem cells.

AMENDED CLAIMS

[received by the International Bureau on 22 July 1997 (22.07.97);
new claim 22 added; remaining claims unchanged (1 page)]

21. A method according to claim 20 wherein the stem cell are hematopoietic stem cells.

22. A method according to claim 21 wherein the dlk is used in conjunction with a cocktail of growth factors for hematopoietic stem cells.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03520

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/18; C07K 1/00
US CL :514/2; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, EPOABS, MEDLINE, CAPLUS, search terms: pg2, pref-1, FA1, dlk, stem cell, differentiation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 5,580,738 A (J. LABORDA) 03 December 1996, see entire document.	1-21
A	LEE et al. *dlk*, *pG2* and *Pref-1* mRNAs encode similar proteins belonging to the EGF-like superfamily. Identification of polymorphic variants of this RNA. Biochim. Biophys. Acta. 1995, Vol. 1261, pages 223-232, see entire document.	1-21
A	LABORDA et al. *dlk*, a putative mammalian homeotic gene differentially expressed in small cell lung carcinoma and neuroendocrine tumor cell line. J. of Biol. Chem. 25 February 1993, Vol. 268, pages 3817-3820, see entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 APRIL 1997	30 MAY 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KENNETH A. SORENSEN